Age and Breeding Effort as Sources of Individual Variability in Oxidative Stress Markers in a Bird Species

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ABSTRACT

Oxidative stress is the imbalance between the production of pro-oxidant substances and the level of antioxidant defenses, which leads to oxidative damage. It has been proposed that senescence is the result of accumulated oxidative damage throughout life. In birds, the sources of individual variability in oxidative stress are still poorly understood. Among these sources, age, as related to senescence, should be particularly relevant. Furthermore, recent findings suggest that breeding effort may also deeply influence susceptibility to oxidative stress. However, there is still no evidence of a link between breeding effort and oxidative damage in any vertebrate. Here we analyzed 288 captive red-legged partridges (*Alectoris rufa*) across a wide age range (i.e., 1–8 yr old), thus including potentially senescent birds. In spite of limitations due to the cross-sectional approach, results revealed that old birds produced less offspring and endured higher levels of oxidized glutathione and peroxidized lipids in erythrocytes than did middle-aged individuals. Old birds also showed higher plasma total antioxidant status and uric acid levels than did younger birds, but lower amounts of circulating carotenoids. Furthermore, hatching success was negatively correlated to lipid peroxidation in females but not in males, supporting the hypothesis that breeding effort promotes oxidative damage.

Introduction

Oxidative stress is defined as the imbalance between the production of pro-oxidant substances (i.e., reactive oxygen species, or ROS) and the state of the antioxidant and repair machinery of the organism, which leads to oxidative damage to biomolecules (Valko et al. 2007). The oxidative stress theory of aging proposes that age-related loss of function (i.e., aging) is the result of accumulated oxidative damage throughout life (Finkel and Holbrook 2000; Buffestein et al. 2008). Therefore, oxidative stress has often been studied in an attempt to understand cell senescence and aging-related diseases in humans (Finkel and Holbrook 2000; Valko et al. 2007).

Cell and molecular biologists have traditionally used mammalian species to answer the question of why we age. However, birds are increasingly being used as model species because they live relatively longer than mammals of similar body masses (Holmes et al. 2001; Holmes and Ottinger 2003). This implies a paradox, given that birds also show an amazingly high metabolism (Holmes and Ottinger 2003; Barja 2007), contradicting the assumption that longevity is inversely related to the rate of oxygen consumption (i.e., Pearl 1928). The study of birds could hence reveal physiological adaptations that would be useful in understanding the aging process. Nevertheless, whereas studies analyzing avian adaptations to aging have mostly focused on interspecific comparisons (Hulbert et al. 2007), much less is known about the sources of individual variability in oxidative stress.

Among factors influencing individual variability, age, as related to the aging process, should play a key role. Studies describing changes in oxidative stress throughout life are abundant in mammalian literature (Oztürk and Gümüşlü 2004; Gil et al. 2006). In contrast, avian reports are mostly limited to the early stages of life because data on aged birds are scarce (e.g., Alonso-Alvarez et al. 2006; Torres and Velando 2007; Vleck et al. 2007). Furthermore, as far as we know, no avian study describes the change in any marker of oxidative damage across the full age range (i.e., from youth to old age; see also the review by Vleck et al. [2007]).

In addition to age, breeding effort could also contribute to the explanation of individual variability in oxidative stress. Surprisingly, although the trade-off between self-maintenance and reproduction has been intimately related to the evolution of aging (Kirkwood and Austad 2000), the influence of breeding effort in oxidative stress has barely been addressed in any species (Monaghan et al. 2009). In fact, the interest of evolutionary biologists in oxidative stress has mostly been directed to sexual selection. In this regard, birds are often used to test the hypothesis where it is suggested that colored sexual secondary traits (many yellow to red traits) produced by carotenoids (i.e., pigments with antioxidant properties) signal the quality of the bearer in terms of resistance to oxidative stress (i.e., von Schantz et al. 1999; see also Hill and McGraw 2006).

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However, birds were the first vertebrates to reveal that breeding effort may imply a cost in terms of oxidative stress. Zebra finches (*Taeniopygia guttata*), whose breeding effort was artificially increased by enlarging their brood size, showed erythrocytes with a high susceptibility to an ROS-induced hemolysis (Alonso-Alvarez et al. 2004*b*; see also Wiersma et al. 2004; Alonso-Alvarez et al. 2006). These studies mostly described a decline in antioxidant defenses with increased effort. Nevertheless, whether this effect leads to oxidative damage remains unknown (see Monaghan et al. 2009).

In this study, the variability in the level of several oxidative stress markers was assessed in 288 captive red-legged partridges (*Alectoris rufa* Linnaeus), ranging from 1 to 8 yr of age (i.e., most of this species' range of age; see "Material and Methods"). In order to assess the impact of breeding effort on oxidative stress, the relationship between these markers and the number of eggs and chicks produced during the preceding season was also analyzed. We predicted that a high investment in reproduction would imply a high level of oxidative damage and low levels of antioxidants. Similarly, we predicted that old birds should show higher levels of oxidative damage and lower levels of antioxidants than would younger individuals. The total antioxidant status (TAS) of plasma (i.e., Miller et al. 1993) and the plasma levels of uric acid and carotenoids were analyzed. Furthermore, the amounts of oxidized and total glutathione (GSSG and tGSH, respectively) were assessed in erythrocytes. Glutathione is a key intracellular antioxidant (Meister and Anderson 1983; Wu et al. 2004), and the oxidized : reduced glutathione couple (GSSG : GSH) is the most important index of redox status within animal cells (Das and White 2002). Finally, the amount of peroxidized lipids in erythrocytes was determined to estimate oxidative damage. Birds were sampled in a single day to reduce potential sources of individual variability.

Material and Methods

The study was performed in a governmental breeding facility at Chinchilla (Albacete, Spain), which maintains a large partridge population obtained from free-living chicks captured on neighboring military land (15,000 ha). Red-legged partridges can live >6 yr in the wild and at least 10 yr in captivity (F. Mougeot, personal communication). They show carotenoid-based red traits (eye rings, beaks, and legs), the redness of which is positively related to circulating carotenoid levels (Alonso-Alvarez et al. 2008; Mougeot et al. 2009).

Birds were housed in couples in outdoor cages (1.6 m × 0.5 m × 0.4 m), which included a shelter with a roof (0.5 m × 0.4 m × 0.3 m) to protect birds during harsh weather conditions. Water and food (commercial pelleted food, Nanta Foods) were provided ad lib. Birds were captured as yearlings to mark them with a ring code indicating the year of birth, but they were not recaptured during life. In this way, although we cannot fully rule out that some stressor associated with captivity may have influenced oxidative stress (e.g., in rats, see Zafir and Banu 2009), we assume that their life conditions were milder than the life conditions of those supported in the wild (unpredictable food and water availability, predators, harsh weather conditions, etc.) and, hence, were less aggressive in terms of oxidative stress. We must also consider that captive animals commonly exhibit longer life spans than do wild individuals.

Birds of different ages were randomly chosen, although all birds at the oldest ages were sampled (sample sizes: 1 yr old = 44, 2 yr old = 46, 3 yr old = 44, 4 yr old = 42, 5 yr old = 48, 6 yr old = 46, 7 yr old = 14, and 8 yr old = 4). Only complete couples were used, thus avoiding birds that had lost their mates during previous months (i.e., differences in behavior and breeding effort). Couples were same-age birds. All birds were blood sampled on the same day (June 8, 2006), that is, at the end of the breeding season. Weather conditions were steady throughout that day.

Blood sampling avoids killing birds and is a common procedure found in the scientific literature for avian species. Most aging studies, however, focus on postmitotic tissues because they accumulate damage throughout life (e.g., neurons, skeletal muscle). Nonetheless, erythrocytes may indicate overall oxidative status because they circulate throughout the body's tissues. They also play a fundamental role in oxygen transport and hence are particularly exposed to oxidative stress (Rizvi and Mauria 2007; Cimen 2008).

Blood samples (0.8 mL) were taken from the brachial vein within 2 min after the removal of the bird from its cage. The cages were randomly distributed across several hectares. Therefore, in most cases, birds were not previously disturbed. Samples were immediately stored at 4°C until centrifugation at 7,200 *g* and plasma separation, which was performed within 10 h. Both plasma and cellular fraction (pellet) were frozen at −80°C until analysis.

Breeding cages were inspected daily. Eggs were removed, identified, and immediately stored at 15°C. At this temperature, embryo development is arrested (Thear 1987). Stored eggs were transferred to incubators (37°C) every 20 d. Six incubation periods (24 d each) were programmed. The number of chicks per cage was determined at hatching time. Synchronizing hatching events was necessary to allow the management of large numbers of chicks in captivity. Red-legged partridges are indeterminate layers. Natural clutch sizes reach 20 eggs, and often two clutches (double nesting) are laid per season (Cramp and Simmons 1980). Hens are able to lay additional clutches if the first is destroyed (Cramp and Simmons 1980). The study was approved by the Spanish Agencia Nacional de Evaluación y Prospectiva.

Antioxidants in Plasma

TAS in plasma was assessed by using commercial kits (Randox Laboratories, Crumlin, UK) adapted to an automated spectrophotometer (A25-Autoanalyzer, Biosystems, Barcelona, Spain). The method is based on the procedure outlined by Miller et al. (1993). Plasma samples were incubated for 15 s with a chromogen composed of metmyoglobin and ABTS (2,2-azino-di-[3-ethylbenzthiazoline sulphonate]). Next, hydrogen peroxide (H_2O_2) was added and the sample was incubated for 195

s. H_2O_2 addition induces the production of the radical cation ABTS, which generates a blue-green color. Color is measured at 600 nm before and after H_2O_2 addition, thus determining the change in color. Any extracellular antioxidant in the plasma sample (e.g., vitamins E and C, uric acid, carotenoids) causes suppression of this color to a degree proportional to their concentration. Results are given as millimoles per liter of total antioxidants in plasma. Repeatability (Lessells and Boag 1987, here and hereafter) was confirmed using a random subset of samples measured twice ($r = 0.96$, $P < 0.001$, $n = 25$; coefficient of variation [CV] $= 0.05$).

Plasma uric acid levels were determined by spectrophotometry, following the uricase/peroxidase method and commercial kits (Biosystems). Uric acid values assessed twice on a subsample were also repeatable ($r = 0.97$, $P < 0.001$, $n = 25$, CV $= 0.04$).

Total carotenoid concentration in plasma was determined by spectrophotometry, using a standard curve of lutein (α-carotene-3,3′-diol; Sigma). High-performance liquid chromatography previously showed that lutein is the predominant carotenoid pigment in the plasma of red-legged partridges (R. Mateo, unpublished data). Plasma aliquots (60 μL) were diluted in acetone (1 : 10) and mixed, and the flocculent protein was precipitated by centrifuging the sample at 11,000 *g* for 10 min. The supernatant was examined in a spectrophotometer (Shimadzu UV-1603, Japan), and the absorbance was determined at 446 nm (Perez et al. 2007). Carotenoid values were assessed twice and were also highly repeatable ($r = 0.99$, $P < 0.001$, $n = 25$, CV $= 0.04$).

Glutathione in Erythrocytes

Glutathione levels were analyzed by following the method described by Griffith (1980) with particular modifications. Briefly, the blood pellet was thawed and the erythrocytes were pipetted avoiding the pellet surface (which contained white blood cells). Erythrocytes were immediately diluted (1 : 10 w/v) and homogenized in a stock buffer (0.01 M PBS and 0.02 M EDTA), always working on ice to avoid oxidation. Three working solutions were created in the same stock buffer as follows: 0.3 mM NADPH (solution I), 6 mM DTNB (solution II), and 50 units of glutathione reductase mL^{-1} (solution III). An aliquot (0.5 mL) of homogenate of blood cells was vortexed with 0.5 mL of diluted trichloroacetic acid (10% in H_2O) three times, for 5 s each time, within a 15-min period. In the meantime, samples were removed from light and refrigerated to prevent oxidation. Afterward, the mixture was centrifuged (1,125 *g* for 15 min at 6°C) and the supernatant was removed. Subsequent steps were performed in an automated spectrophotometer (A25-Autoanalyzer, Biosystems). Solutions I and II were mixed at a 7 : 1 volume, respectively. One hundred sixty μL of this new mixture was automatically added to 40 μL of sample (i.e., supernatant) in a cuvette. Afterward, 20 μL of solution III was added after 15 s, and the absorbance at 405 nm was monitored after 30 and 60 s. The change in absorbance was used to determine tGSH levels by comparing the output with the results from a standard curve generated by serial dilution of glutathione from 1 mM to 0.031 mM. Results are given in millimoles per gram of pellet.

To determine GSSG, an aliquot (400 μL) of the supernatant obtained for tGSH assessment was adjusted to a pH of 7.5 by adding 6 N NaOH. Afterward, 2-vinilpiridine (8 μL) was added to the aliquot, and the mixture was vigorously shaken at ambient temperature and in the dark to promote glutathione derivatization. The mixture was then centrifuged (3,500 rpm for 10 min), and the change in absorbance of the supernatant was assessed at 405 nm as described for the tGSH assay. GSSG and tGSH levels assessed twice were repeatable ($r = 0.92$ and 0.89, respectively, both $P < 0.01$, $n = 25$, CV < 0.07).

Lipid Peroxidation in Erythrocytes

Lipid peroxidation was assessed following Aust (1985). The principle of the test is based on the fact that most tissues contain a mixture of thiobarbituric acid–reactive substances (TBARS), including lipid hydroperoxides and aldehydes, whose concentrations increase because of oxidative stress. One mL of the same homogenate used for tGSH analyses was mixed with 2 mL of a solution (trichloroacetic acid 15%, HCl at 0.25 N, and thiobarbituric acid 0.375%, all in H_2O) and 20 μL of diluted BHT ([2,6-di-tert-butyl-4-methylphenol] at 2% in ethanol), all in closed glass tubes. Tubes were then warmed for 30 min at 90°C and afterward cooled on ice (10 min). The supernatant absorbance was measured by spectrophotometry at 535 nm after centrifugation (2,025 *g* for 15 min). Concentrations of peroxidized lipids were determined by comparing absorbances to those obtained from a curve with 0, 1.25, 2.50, and 5 nmol mL^{-1} of malondialdehyde (MDA) in H_2O (i.e., end product of lipid peroxidation; Aust 1985). Levels of peroxidized lipids were expressed as nanomoles of MDA per gram of pellet. TBARS values assessed twice were repeatable ($r = 0.85$, $P = 0.03$, $n = 25$, CV $= 0.07$).

Although often used as a marker of oxidative damage, the specificity of the TBARS method has been questioned because of a potential interference of MDA amounts in the diet (Monaghan et al. 2009). Nonetheless, although diet composition may alter lipid profile in partridges (Surai et al. 2001*a*), in our captivity conditions with a standardized diet, any possible alteration would affect all birds equally. Moreover, that sampling time was not related to TBARS variability (see below) suggests that circadian rhythms in food intake (i.e., high intake at early and late hours) did not affect the results.

Statistical Analyses

The numbers of eggs and chicks as well as the hatching success (number of chicks divided by number of eggs) obtained by each couple were adjusted to a Poisson distribution and tested by generalized linear models (PROC GENMOD in SAS software; SAS Institute 2001). Age was considered as a continuous variable to allow testing for different trends (age-related increases, decreases, or quadratic relationships). Age and squared age (age^2) of the couple were introduced as covariates. The

quadratic adjustment is appropriate in cross-sectional studies because it reveals those patterns otherwise masked by simpler linear adjustments.

Generalized mixed linear models (PROC GLIMMIX in SAS) were performed to analyze oxidative stress markers and antioxidants. Normality was met in all dependent variables. Because birds forming a couple were placed in the same cage and shared the same breeding cycles and environment (e.g., shadow, temperature, humidity), the identity of the cage was fixed as a random factor in all the models (range of P values: 0.15–0.005). The age and the squared age (age^2) of the bird were tested as covariates. Sex (a fixed factor), sampling hour, squared sampling hour, and reproductive variables (covariates) were also added. To disentangle the covariation among oxidative stress markers, the remaining blood variables were also tested as covariates in the models. The inclusion of tGSH as a covariate in the model testing GSSG was used to estimate variability in the GSSG : GSH couple, avoiding the assumptions associated with the use of ratios (i.e., slope = 1, intercept = 0, same error; Packard and Boardman 1999). Interactions between sex and covariates were tested. Values provided for each sex were least squares means and standard errors from the models (PROC LSMEANS in SAS). Slopes ± SE of the relationship between dependent variables and covariates were obtained from the mixed models. Explained deviance for the effect of covariates on dependent variables is provided. Different procedures for selecting the best-fitted model were used (forward and backward stepwise procedures and the Akaike Information Criterion), and they all reported similar results. For simplicity, only the results of backward procedures were shown, thus removing all nonsignificant terms at $P > 0.05$. To allow for the interpretation of age-related patterns, circles in Figures 2–4 represent residuals obtained from the models and were corrected by adding the mean value in the population (i.e., mean-adjusted residuals).

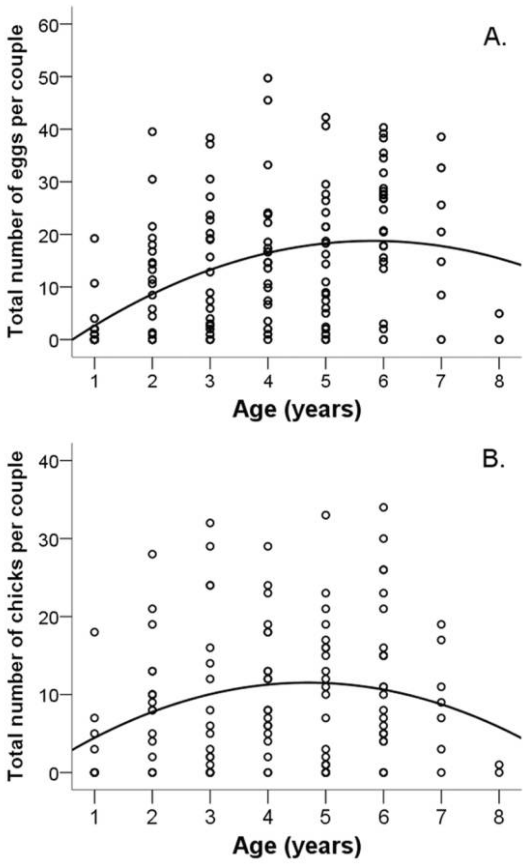

Figure 1. Reproductive senescence in red-legged partridges. Total number of eggs (*A*) and total number of chicks (*B*) produced by couples of different ages. Circles represent raw values and lines represent quadratic adjustments (see "Results").

Results

Reproductive Senescence

The number of eggs produced during the breeding season showed a quadratic relationship with the age of the couple (age: $\chi^2 = 428.9$, df = 1, $P =< 0.0001$, slope = $+0.881 \pm 0.047$; age^2: $\chi^2 = 253.5$, df = 1, $P < 0.0001$, slope = -0.080 ± 0.005; explained deviance = 21%; Fig. 1*A*). Young and old couples laid fewer eggs. The same pattern was found in the number of chicks (age: $\chi^2 = 261.9$, df = 1, $P =< 0.0001$, slope = $+0.882 \pm 0.059$; age^2: $\chi^2 = 181.2$, df = 1, $P < 0.0001$, slope = -0.089 ± 0.007; explained deviance = 12.3%; Fig. 1*B*). The influence of age on hatching success was not detected (both age terms $P > 0.27$).

Plasma Antioxidants

In the TAS model, age and sex showed significant effects ($F_{1,138} = 6.82$, $P = 0.010$, and $F_{1,138} = 4.50$, $P = 0.036$, respectively). Age was positively related to TAS (slope = $+0.015 \pm 0.007$), and males showed lower TAS levels than did females (males: 1.26 ± 0.13 mmol L^{-1}; females: 1.31 ± 0.13 mmol L^{-1}). Uric acid ($F_{1,138} = 96.7$, $P < 0.001$), carotenoids ($F_{1,138} = 8.82$, $P = 0.004$), and TBARS values ($F_{1,138} = 4.78$, $P = 0.031$) also showed significant relationships with TAS. The first two were positively associated with TAS (slopes = $+0.060 \pm 0.005$ and $+0.010 \pm 0.003$, respectively), whereas the TBARS covariate showed the opposite pattern (slope = -0.037 ± 0.021). The influence of age remained when all covariates and sex were removed from the model ($F_{1,142} = 8.69$, $P = 0.004$, slope = $+0.025 \pm 0.008$; explained deviance = 7.8%; Fig. 2*A*).

Similarly, uric acid values showed a significant positive relationship with age and circulating carotenoids ($F_{1,140} = 11.48$, $P = 0.001$, slope = $+0.248 \pm 0.072$, and $F_{1,140} = 12.60$, $P = 0.001$, slope = $+0.136 \pm 0.032$, respectively). Uric acid values also differed between sexes ($F_{1,140} = 4.59$, $P = 0.034$; males: 6.10 ± 0.31 mg dL^{-1}; females: 6.65 ± 0.31 mg dL^{-1}). The influence of age persisted when tested alone ($F_{1,142} = 9.87$, $P = 0.002$, slope = $+0.225 \pm 0.072$; explained deviance = 4.3%; Fig. 2*B*).

In contrast, carotenoid concentration was negatively related to age ($F_{1,137} = 5.67$, $P = 0.019$, slope = -0.288 ± 0.136) but positively associated with uric acid ($F_{1,137} = 13.74$, $P < 0.001$,

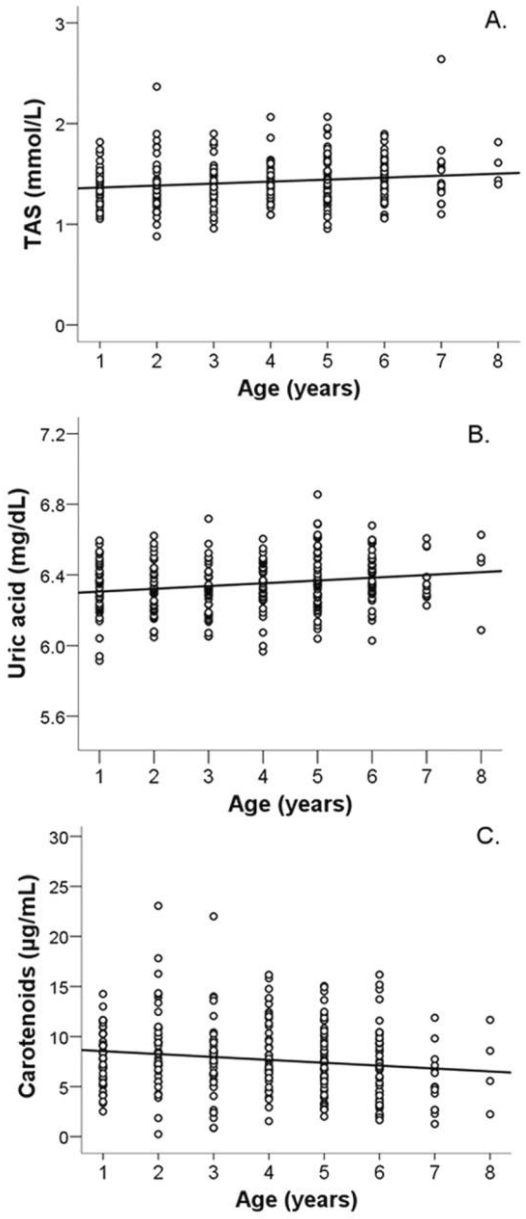

Figure 2. Relationship between plasma antioxidants and age. Total antioxidant status (*TAS*; *A*), uric acid levels (*B*), and carotenoid levels (*C*) in plasma. *Y*-axis represents residuals of the models corrected by mean values in the population. Linear adjustments were also obtained from the models (see "Results").

slope $= +0.434 \pm 0.109$) and GSSG ($F_{1,137} = 10.21$, $P = 0.002$, slope $= +2.99 \pm 0.94$). This result was also obtained when the GSSG : tGSH ratio was tested instead of GSSG ($P = 0.03$). A significant interaction between hatching success and sex was detected ($F_{1,137} = 4.52$, $P = 0.036$; explained deviance: 17.4%) with females but not with males, showing a significant positive correlation ($r = +0.21$, $P = 0.02$, and $r = +0.04$, $P = 0.65$, respectively). The relationship between carotenoids and age remained when all covariates and sex were removed ($F_{1,142} = 9.32$, $P = 0.003$, slope $= -0.35 \pm 0.13$; explained deviance $= 3\%$; Fig. 2*C*).

Glutathione in Erythrocytes

GSSG levels showed a quadratic relationship with age (age: $F_{1,138} = 8.53$, $P = 0.004$, slope $= -0.091 \pm 0.031$; age^2: $F_{1,138} = 5.58$, $P = 0.020$, slope $= +0.010 \pm 0.004$), with the sex factor being significant ($F_{1,138} = 15.82$, $P < 0.001$; males: 0.35 ± 0.04 μmol g^{-1}; females: 0.29 ± 0.04 μmol g^{-1}). In the same model, TBARS and tGSH were positively related to GSSG ($F_{1,138} = 9.72$, $P = 0.002$, slope $= +0.012 \pm 0.004$, and $F_{1,138} = 4.31$, $P = 0.041$, slope $= +0.025 \pm 0.012$, respectively). The relationship between GSSG and age remained when all covariates and sex were removed (age: $F_{1,141} = 9.57$, $P = 0.002$, slope $= -0.097 \pm 0.031$; age^2: $F_{1,141} = 6.74$, $P = 0.010$, slope $= +0.010 \pm 0.004$; explained deviance $= 3.2\%$; see Fig. 3*A*).

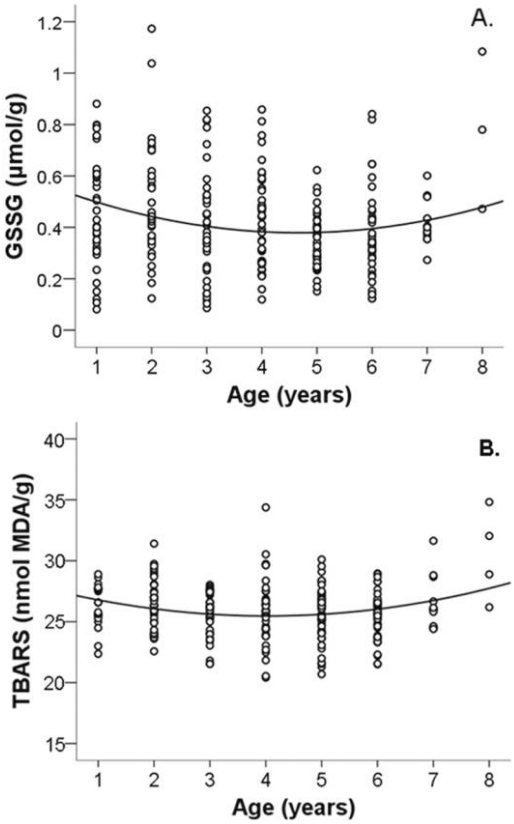

Figure 3. Relationship between two markers of oxidative stress and age. Oxidized glutathione (*GSSG*; *A*) and lipid peroxidation levels (*B*) per gram of packed erythrocytes. Lipid peroxidation was assessed with the thiobarbituric acid–reactive substances (TBARS) procedure and expressed as malondialdehyde concentration (*MDA*; end product of lipid peroxidation). *Y*-axis represents residuals of the models corrected by mean GSSG and TBARS values in the population. Linear adjustments were also obtained from the models (see "Results").

Lipid Peroxidation in Erythrocytes

TBARS also showed a quadratic pattern with age (age: $F_{1,109} = 8.50$, $P = 0.004$, slope $= -0.767 \pm 0.433$; age^2: $F_{1,109} = 8.67$, $P = 0.004$, slope $= +0.108 \pm 0.055$; explained deviance $= 4.5\%$). Partridges at intermediate ages showed lower lipid peroxidation in their erythrocytes. Sampling time and its squared term were not related to TBARS variability (both $P > 0.45$). The interaction between sex and hatching success was significant and was retained in the model (sex: $F_{1,109} = 5.49$, $P = 0.021$; hatching success: $F_{1,109} = 0.10$, $P = 0.757$; interaction: $F_{1,109} = 5.79$, $P = 0.018$; explained deviance $= 12\%$). In other words, whereas TBARS was not significantly associated with hatching success in males ($r = -0.16$, $P = 0.142$), a significant correlation was detected in females ($r = +0.33$, $P = 0.002$; see Fig. 4). This suggests that females able to produce eggs with higher hatching probabilities suffered higher lipid peroxidation in their erythrocytes. The TBARS model was repeated with only the age covariates, and the same pattern was observed (age: $F_{1,141} = 5.03$, $P = 0.027$, slope $= -0.769 \pm 0.433$; age^2: $F_{1,141} = 5.73$, $P = 0.018$, slope $= +0.107 \pm 0.054$; explained deviance $= 4\%$; i.e., Fig. 3*A*).

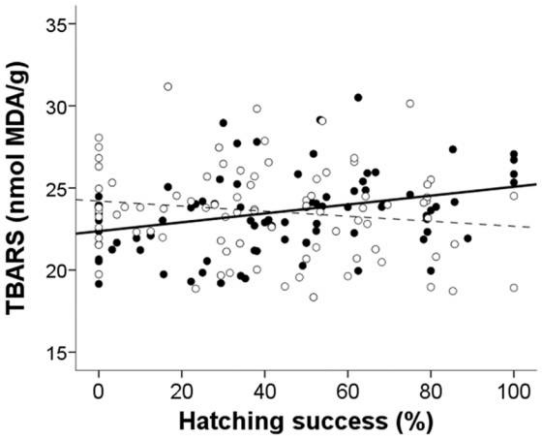

Figure 4. Relationship between lipid peroxidation in erythrocytes and hatching success. Filled circles and solid line represent data from females, and open circles and dashed line represent data from males. *Y*-axis shows residuals of a model, including the age of the birds and corrected by the mean TBARS (thiobarbituric acid–reactive substances) value in the population. Linear adjustments were also obtained from the model (see "Results"). *MDA* = malondialdehyde.

Discussion

Our cross-sectional approach revealed that old birds have significantly higher TAS and uric acid plasma levels than do younger birds but lower values of plasma carotenoids. Results also showed that both young and old birds sustained higher GSSG and TBARS levels than did middle-aged birds. Furthermore, the study provides evidence of the cost of breeding effort in terms of oxidative damage. We must stress that the effects were statistically significant even though birds likely experienced mild conditions during their lifetime (see "Material and Methods"). Nonetheless, the magnitude of these effects was relatively low.

The different shape of the relationships between several blood parameters and age was particularly intriguing. Whereas GSSG and TBARS levels described a quadratic pattern, TAS and uric acid and carotenoid levels showed linear relationships. This difference could be due to the sampling design. Cross-sectional studies imply that individuals in the first age classes should include weak individuals, which are subsequently filtered by selective mortality (Nussey et al. 2008). In young partridges, high TBARS and GSSG values and low TAS and uric acid levels support such a hypothesis. It should be noted that, while a longitudinal approach could have avoided this effect, it would have required that birds be monitored over an 8-yr period.

In old birds, the relatively high GSSG and TBARS levels in addition to the decline in reproductive output (Fig. 1*A*, 1*B*) suggest that, in spite of probable mild captivity conditions and selection for survivorship throughout life, old partridges were in fact senescent individuals. This is the first report of a difference between middle-aged and old birds in the levels of these compounds. Higher GSSG levels in old birds could reflect an imbalance in glutathione metabolism, such as was previously reported in senescent mammals (Oztürk and Gümüşlü 2004; Voss and Siems 2006; Toroser and Sohal 2007). Moreover, because lipid peroxidation seems to determine cell membrane integrity and hemolysis (see Brzezinska-Slebodzinska 2001; Armutcu et al. 2005), high TBARS levels in old partridges would agree with longitudinal data from zebra finches, which showed an age-related decline in erythrocyte resistance to ROS-induced hemolysis (Alonso-Alvarez et al. 2006).

In terms of plasma antioxidants, TAS and uric acid levels increased with age, contradicting our predictions. These results could be explained by (1) selective mortality in the population (i.e., old birds are "better" birds) or (2) the presence of a compensatory mechanism (i.e., old birds are challenged birds). Lower carotenoid concentration and higher TBARS and GSSG levels in old birds support the second explanation. In greenfinches (*Carduelis chloris*), the induction of an inflammatory response that produced free radicals (i.e., Bertrand et al. 2006) promoted a rise in plasma TAS (Hõrak et al. 2007). In domestic chickens (*Gallus gallus domesticus*), corticoid-induced stress increased plasma lipid peroxidation (Lin et al. 2004*a*, 2004*b*) as well as TAS and uric acid levels (Lin et al. 2004*a*, 2004*b*; Maurice et al. 2007). Here, a similar adaptive response could have developed but as a result of oxidative challenges derived from aging. Nevertheless, most mammalian studies report age-related declines in TAS (in serum and in different tissues with similar assay techniques; Quiles et al. 2004; Li et al. 2007; Mendoza-Nunes et al. 2007). Similarly, the parallel increase of uric acid levels is not well supported by human studies, which report decreases, increases, or no change with age (Voss and Siems 2006). Although uric acid in blood may contribute to controlling oxidative stress (Ames et al. 1981; Machin et al. 2004; Costantini 2008; but see Cohen et al. 2007, 2008), its role in avoiding lipid peroxidation could be minor because it is, in fact, a hydrosoluble compound (Voss and Siems 2006). Moreover, uric acid levels in plasma can be influenced by the protein content in the diet (Voss and Siems 2006; Smith et

al. 2007) or by protein catabolism associated with starvation (e.g., Alonso-Alvarez and Ferrer 2001). Here, nonetheless, food availability and diet composition were standardized.

In terms of carotenoids, this is the first report of a relationship between circulating carotenoids and age from youth to old age in any bird species. Results agree with recent studies reporting a decline in the expression of carotenoid-dependent sexual secondary traits with age in this (Alonso-Alvarez et al. 2009) and other (Torres and Velando 2007) avian species. The lower plasma levels in old partridges suggest a gradual oxidation of these pigments. In fact, circulating carotenoids in birds decline in the presence of high oxidative stress (Alonso-Alvarez et al. 2004*a*; Hõrak et al. 2007; but see Isaksson and Andersson 2008). However, whether carotenoids have an active role as antioxidants in birds or whether they are simply passively oxidized by free radicals has been much debated (Hartley and Kennedy 2004; Costantini and Møller 2008). In our case, carotenoid values were positively related to TAS, and the same relationship was detected in other independent studies in red-legged partridges (Alonso-Alvarez et al. 2008, 2009; see also Perez-Rodriguez et al. 2008). Furthermore, carotenoid levels were positively associated with GSSG, suggesting that carotenoids could be actively mobilized to fight off intracellular oxidative stress.

With regard to the cost of reproduction in terms of oxidative stress, the first evidence in any species came from studies of insects. The exposure of fruit flies (*Drosophila melanogaster*) to a pro-oxidative substance (Paraquat) led to a faster mortality among flies whose reproduction was stimulated by means of nutrition or hormone manipulation (Salmon et al. 2001; Wang et al. 2001). In vertebrates, the first reports were of zebra finches whose breeding efforts were artificially increased by enlarging brood size, which led to a decline in the resistance to ROS-induced hemolysis (Alonso-Alvarez et al. 2004*b*; see also Wiersma et al. 2004). However, studies of fruit flies involved far from natural manipulations (Paraquat is an herbicide), whereas zebra finch studies mostly assessed the status of the antioxidant machinery. Probably a less ambiguous way to detect the imbalance between ROS production and antioxidants would be to assess damage to biomolecules (Costantini 2008; Monaghan et al. 2009). In this sense, it has recently been claimed that it is not known whether breeding effort produces oxidative damage (Monaghan et al. 2009). Although our results are not experimental, they suggest that such damage may indeed be present in female partridges.

Female partridges with higher hatching success throughout the breeding season were also those that sustained higher plasma carotenoid levels at the end of reproduction. If we assume that carotenoid levels after the period of breeding effort may reflect carotenoid levels at the laying period, then this result would indicate that hatching success depends on the female's capacity to allocate carotenoids to the egg yolk, thereby protecting the embryo from oxidative stress (Blount et al. 2000; Surai et al. 2001*b*). Experimental increases in carotenoid availability in food led to higher yolk-carotenoid levels and better hatchability in some bird and fish species (McGraw et al. 2005; Ahmadi et al. 2006) but not in others (Remes et al. 2007; Grether et al. 2008). In red-legged partridges, a negative correlation between circulating carotenoids before laying and clutch size has been reported (Bortolotti et al. 2003). Unfortunately, these authors did not analyze hatching success. The allocation of antioxidants to eggs could have involved a trade-off with self-maintenance, leading to oxidative damage in the mother's tissues. Nonetheless, because carotenoid content in egg yolk was not assessed, this hypothesis cannot be validated. We must also consider that egg-laying capacity was possibly stimulated to its limit. We can only speculate on how these findings can be extrapolated to wild partridges, which would be subject to other energy requirements (foraging, nest defense, etc.).

In summary, this study reports, for the first time, the age-related variability in circulating levels of several oxidative stress markers across the full age range of an avian species, that is, including potentially senescent birds. In spite of limitations due to the cross-sectional approach and captivity conditions, the study reveals that old birds may suffer an imbalance in glutathione metabolism as well as high lipid peroxidation, such as was previously described in senescent mammals. The study also provides the first correlational support for a link between breeding effort and oxidative damage. Nonetheless, further experimental approaches are required to confirm this finding.

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